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(54) **Title:** METHODS FOR PRODUCTION OF THERAPEUTIC CYTOKINES

(57) **Abstract**

Recent work had disclosed that tryptophan–containing dipeptides have immunomodulatory activity *in vivo*. In this invention, it is disclosed that the dipeptides have activity *ex vivo* and $CD2^+$ cells can be induced to differentiate into Th1 cells, thus expressing Th1–associated cytokines. These cytokines are useful as a pharmaceutical agent and in fact because of the complexity of the cocktail of cytokines, are more active against a pathological condition. Embodiments of this invention are directed to the tailoring of therapy possible by the removal of $CD2^+$ cells from a subject suffering from a pathological condition and contacting the cells *ex vivo* and the dipeptide compounds to induce the cells to synthesize Th1–associated cytokines. For treatment of the pathological condition, either the cocktail or the contacted cells can be administered to the subject.

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METHODS FOR PRODUCTION OF THERAPEUTIC CYTOKINES

CROSS-REFERENCES TO RELATED APPLICATIONS

This application claims the benefit of the filing dates of U.S. Provisional Patent applications, 60/080,615, filed April 3, 1998 and 60/094,422, filed July 28, 1998. It is also related to U.S. Patent application 09/055,092, filed April 3, 1998 (Green and Sinackevich), and U.S. Patent application 09/055,051, filed April 3, 1998 (Green and Sinackevich).

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

Not Applicable.

BACKGROUND OF THE INVENTION

Cytokines, including interferon, are used in many different pharmaceutical applications. They have been proven to be factors in infection, cancer and inflammation. Cytokines, however, are a class of proteins with individual members playing different roles in immunomodulation. Because the different cytokines have different specificity, the few individual cytokines that are currently available do not treat all conditions in which cytokine therapy would be beneficial.

CD2 is found on all human peripheral T lymphocytes, thymocytes, approximately 50% of thymic B cells, 9% to 12% of bone marrow cells, and the majority of CD3- NK cells (FUNDAMENTAL IMMUNOLOGY, 3RD ED., Paul, (ed.), Raven Press, New York (1993)). CD2 binds primarily to CD58 on antigen-presenting cells and induces costimulatory signals in T-cells. Its cellular function is in T-cell activation, T or NK-mediated cytolysis, induction of apoptosis in activated peripheral T-cells, regulation of T-cell anergy and production of cytokines by T-cells. It is a member of the Ig superfamily and is found naturally as a dimer with each chain having a molecular weight of 50 kD.

Prothymocytes ultimately differentiate into two types of T-cells; T helper or $CD4^+$, and cytotoxic T lymphocytes (CTL) or $CD8^+$ cells. T helper cells are further divided into two classes, Th1 and Th2. Th1 cells are involved in cell-mediated immunity. They are largely responsible for delayed-type hypersensitivity, are able to lyse virally-

infected cells and stimulate macrophages to destroy pathogens. Th1 cells also contribute to the pathogenesis of autoimmune disease. Th2 cells, on the other hand, appear to inhibit autoimmune disease development (Adorini, *et al., Autoimmunity* **23**:53 (1996)). Th2 cells also are intimately involved in humoral immunity, providing growth and differentiation factors for B cells.

Th1 and Th2 cells exert their activity through the cytokines they express. Th1 cells express interleukin 2 (IL-2), γ-interferon (γ-IFN) and tumor necrosis factor β (lymphotoxin or TNFβ). Th2 cells express interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6) and interleukin 10 (IL-10). In addition to their action on other cells, the cytokines expressed by Th1 and Th2 cells also affect each other. The cytokines released by Th1 cells are inhibitory to Th2 cells and vice versa. Therefore, by enhancing the action of one subset of T-cells, the action of the other subset is diminished.

The development of Th1 and Th2 cells from precursor Th0 cells depends on a variety of factors, including the environment, dose of antigen and nature of the immunogen. It is believed that interleukin 12 (IL-12) and γ-IFN are necessary for Th1 development while IL-4 is necessary for Th2 development (Del Prete, *Int'l Rev. Immunol.* **16**:427 (1998)).

There is a need in the art for therapeutic and prophylactic treatments which drive cell-mediated immunity and suppress humoral immunity. For example, it is hypothesized that HIV vaccines have failed largely because the vaccines are designed to stimulate humoral immunity. However, if the T-cells of a person being vaccinated are primed to differentiate into Th1 cells, that person will mount a cell-mediated response to HIV-infected cells. Alternatively, in many cancers and pathogenic infections, IL-6, along with tumor necrosis factor α (TNFα) and interleukin 1 (IL-1), are overexpressed by, among other cells, Th2 cells. This leads to the well known pathological conditions of pneumonia and septic shock. Pharmaceutical compositions which drive cell-mediated immunity and suppress humoral immunity would prevent these pathological conditions.

L-Glu-L-Trp, also known as thymogen, is a dipeptide known normalize immune system function. The drug was found to be the active principle in an extract of the thymus gland called thymosin. (Morozov et al., United States patent 5,070,076.) The dipeptide has been shown to be effective in the treatment of immunodeficient, immunodepressed or hyperactive immune states. (Khavinson et al., WO 92/17191; Khavinson et al., WO 95/03067; and Morozov et al., United States patent 5,538,951.) Pro-drugs of L-Glu-L-Trp, such as cyclized versions of the dipeptide or linear polymers

of the dipeptide, are processed by the body into the active compound. (Khavinson et al., WO 93/08815.) Two related compounds, L-Ile-L-Trp and L-Leu-L-Trp, also have been shown to have immunomodulating properties similar to L-Glu-L-Trp. (Khavinson et al., WO 94/20063.) In addition to these properties, L-Glu-L-Trp has anti-angiogenic activity. (Green et al., WO 97/12625.)

SUMMARY OF THE INVENTION

We have discovered a more effective pharmaceutical composition; one that would more accurately mimic the response of the immune system to foreign antigens. This composition is a cocktail of cytokines with the same relative amounts of cytokines as in a subject undergoing immune challenge. Because some cytokines involved in the immune response have yet to be discovered and because of the complexity of the immune response, such a cocktail is practically impossible to produce from purified and/or recombinant cytokines. In our invention, we have obtained such a cocktail of cytokines from the source; CD2-bearing T-cells.

We have discovered that tryptophan-containing compounds, when contacted to $CD2^+$ cells, drives the $CD2^+$ cells down the Th1 pathway, thereby upregulating cytokines associated with the Th1 pathway, including but not limited to, interleukin 2, α-interferon and γ-interferon. In addition, by upregulating cytokines associated with the Th1 pathway, contact with the tryptophan-containing compounds of this invention inhibits the formation of cells along the Th2 pathway, thus downregulating cytokines associated with this pathway, including but not limited to, interleukin 4, interleukin 5, interleukin 6 and interleukin 10. Upregulation of cytokines associated with the Th1 pathway comprises contacting a cell culture of $CD2^+$ cells with an amount of a tryptophan-containing compound effective to increase interferon production. The tryptophan-containing compounds of this invention are selected from the group consisting of:

(1) L-Xaa-L-Trp;

(2) a cyclic form of L-Xaa-L-Trp;

(3) a linear or cyclic polymer of L-Xaa-L-Trp, the polymer having no more than 20 or no more than 10 amino acids; and

(4) a derivative of any of the foregoing compounds which hydrolyses in aqueous solution into any of the foregoing compounds, and

wherein Xaa is Glu, Ile or Leu.

In different embodiments of the invention, the cell culture initially comprises Th0, Th1, Th2 or NK cells and after contacting with L-Xaa-L-Trp, the cell culture comprises a higher percentage of Th1 cells. If the cell culture already comprises Th1 before adding L-Xaa-L-Trp, after adding the dipeptide, the cell culture comprises a higher percentage of over-functionalized Th1 cells. In these embodiments, the $CD2^+$ cells are from bone marrow or peripheral blood. In one aspect of this embodiment, the bone marrow or peripheral blood cells are enriched for $CD2^+$ cells.

In another aspect of this embodiment, the effective amount of L-Xaa-L-Trp per $CD2^+$ cell is from about 1 to about 4×10^6 molecules. The L-Xaa-L-Trp can be, among other compounds, L-Glu-L-Trp or L-Ile-L-Trp.

Another embodiment of this invention provides for a method of producing a composition comprising a cocktail of cytokines associated with the Th1 pathway comprising contacting a cell culture comprising $CD2^+$ cells with an amount of L-Xaa-L-Trp effective to increase interferon production and collecting a supernatant comprising the cocktail of cytokines from the cell culture. In one aspect of this invention, the cell culture comprises cells taken from healthy individuals.

In yet another embodiment of this invention, a composition is provided which comprises a cocktail of cytokines produced by contacting a cell culture comprising $CD2^+$ cells with an amount of L-Xaa-L-Trp effective to increase production of cytokines associated with the Th1 pathway and decrease production of cytokines associated with the Th2 pathway, collecting supernatant from the culture. The cocktail is then characterized by having at least about 25% greater activity in the woodchuck hepatitis model than an equivalent amount of a purified interferon. In a preferred aspect of this embodiment, the cocktail has an activity of at least about 50% in the woodchuck hepatitis model. In another aspect of this embodiment, the cocktail is combined with a pharmaceutically acceptable carrier. In an alternative aspect, the cocktail is lyophilized.

In another embodiment, this invention provides a composition comprising a cocktail of cytokines which, compared with a cocktail produced by cultured, untreated peripheral white blood cells, comprises a statistically significant increase in the amount of at least one cytokine associated with the Th1 pathway and a statistically significant decrease in the amount of at least one cytokine associated with the Th2 pathway.

One other embodiment of this invention provides for a method for treating a subject suffering from a pathological condition responsive to interferon treatment. Such pathological conditions include, but are not limited to, viral infection, bacterial infection,

fungal infection, benign tumor, malignant tumor, autoimmune disease, degenerative inflammatory condition and asthma. The method of treatment first comprises producing a cocktail of cytokines produced by contacting a cell culture of $CD2^+$ cells with an amount of L-Xaa-L-Trp effective to increase production of cytokines associated with the Th1 pathway and decrease production of cytokines associated with the Th2 pathway. Secondly, the method comprises collecting the supernatant comprising the cocktail of cytokines from the cell culture and characterizing it by having at least about 25% greater activity in the woodchuck hepatitis model than an equivalent amount of a purified interferon. Finally, a pharmacologically effective amount of the cocktail of cytokines is administered to the subject. In one aspect of this embodiment, the cell culture comprises cells taken from the subject, preferably a human. In another aspect, the cell culture comprises cells taken from another subject who does not suffer from the pathological condition.

Another embodiment of this invention provides for a method of treating a subject suffering from a pathological condition including, but not limited to, viral infection, bacterial infection, fungal infection, benign tumor, malignant tumor, autoimmune disease, degenerative inflammatory condition and asthma. The method of treatment comprises contacting a cell culture comprising $CD2^+$ cells with an amount of L-Xaa-L-Trp effective to increase production of cytokines associated with the Th1 pathway and decrease production of cytokines associated with the Th2 pathway and administering a pharmacologically effective amount of the cell culture to the subject. In one aspect of this embodiment, the cell culture comprises cells taken from the subject, preferably from bone marrow or peripheral blood. In another aspect of this invention, the subject is immunosuppressed. However, the cell culture can comprise cells taken from an individual who does not suffer from the condition. In an alternate aspect of this embodiment, the cell culture is encapsulated.

In yet another embodiment, a method for identifying an optimal therapeutic regimen for a subject with a tumor is provided. The method comprises cocultivating a plurality of cultures of tumor cells from said subject and a population of white blood cells comprising $CD2^+$ cells from said subject; contacting each of the plurality of cultures with a different regimen of an L-Xaa-L-Trp compound of this invention; measuring the rate of growth of the tumor cells in each the cultures; and determining the regimen that provides the optimal therapeutic regimen whereby said optimal therapeutic regimen is characterized by inhibition of growth of said tumor cells.

In a preferred aspect of this embodiment, the subject is a human and the tumor cells are from a solid tumor. In another aspect of this embodiment, the tumor cells are hematopoietic in origin. In another aspect of this embodiment, the tumor cells and white blood cells are separated by a membrane permeable to compounds with a molecular weight of less than about 100,000 Daltons. In another aspect of this embodiment, the white blood cells are from the bone marrow. In yet another aspect of this embodiment, at least about 5 differing amounts of L-Xaa-L-Trp are added to the plurality of cultures of tumor cells and $CD2^+$ cells, preferably about once per day. In a preferred aspect, the tumor cells and $CD2^+$ cells are cocultivated for at least about 5 days and the inhibition of tumor cell culture growth is at least about 3-fold.

DEFINITIONS

Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton, *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York (1994), and Hale & Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY (1991) provide one of skill with a general dictionary of many of the terms used in this invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, amino acid sequences are written left to right in amino to carboxy orientation, respectively. The headings provided herein are not limitations of the various aspects or embodiments of the invention which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole.

The phrase "amino acid" refers to both the 20 naturally occurring amino acids as well as the non-naturally occurring amino acids and derivatives of the naturally occurring amino acids. Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission.

The term "tryptophan-containing compound" refers to a polypeptide of at least two amino acids, one of which is tryptophan. The tryptophan-containing compounds useful in this invention have immunomodulatory activity in that, when in

contact with $CD2^+$ cells, the tryptophan-containing compounds drive the $CD2^+$ down the Th1 pathway. In a preferred embodiment, the tryptophan-containing compound is "L-Xaa-L-Trp." L-Xaa-L-Trp refers to a dipeptide comprising at least two L-amino acids linked by a peptide bond. The second amino acid is Trp. The first amino acid can be one of any amino acids but is preferably either Glu, Ile or Leu. The most preferable amino acid is Glu. The term "L-Glu-L-Trp" refers to a dipeptide comprising Glu and Trp linked by a peptide bond. The phrase "cyclic form" refers to the tryptophan-containing compound existing in equilibrium between linear and cyclized states, equilibrium favoring the linear state. For example, in the blood, cyclic tryptophan-containing compounds would tend to equilibrate into the linear form. Regarding "linear or cyclic polymers" of tryptophan-containing compounds, once introduced into the body, these compounds undergo proteolytic degradation, thereby releasing the most active form of the compound, L-Xaa-L-Trp.

The phrase "cell culture" refers to a population of mammalian cells in an *in vitro* environment. The maintenance of such cells is well-known to those of skill in the art and is described in Freshney, CULTURE OF ANIMAL CELLS: A MANUAL OF BASIC TECHNIQUE, 3RD ED., Wiley-Liss (1994). The phrase "bone marrow cells" refers to cells removed from the bone marrow of a subject.

The phrase "peripheral blood cells" refers to cells removed from a blood sample taken from, e.g., a vein, typically through venipuncture. The phrase "cells taken from a subject" refers to the removal of hematopoietic cells from a subject. The cells can be removed from the blood, *i.e.*, peripheral blood cells, from bone marrow, *i.e.*, bone marrow cells, or from any other source of white blood cells, particularly T-cells. The phrase "white blood cells" refers to nucleated cells found in the hematopoietic system. Thus, white blood cells include lymphocytes, macrophages, eosinophils, neutrophils and other mononuclear blood cells.

The phrase "cocultivating a plurality of cultures" refers to maintaining simultaneously more than one cell culture descended from one larger culture. Typically, this is done by splitting a larger cell culture into several smaller cultures and seeding the new cultures in wells of tissue culture plates, *e.g.*, 6-well, 8-well, 12-well or 48-well plates from Corning.

The phrase "$CD2^+$ cells" refers to white blood cells which express CD2 on their cell surface. This subgroup of cells includes prothymocytes and cells derived from prothymocytes, *e.g.*, T-cells and NK cells. The phrase "enriched for $CD2^+$ cells" refers to

selecting for a $CD2^+$ subgroup of cells based on the presence of CD2 on the cell surface. Selection for $CD2^+$ cells can be accomplished by utilizing an antibody that recognizes CD2. Alternatively, $CD2^+$ cells can be identified by the CD2 ligand or an antibody directed against CD2 bound to a solid phase. CD2 cells include human cells and cells of other animals that bear a functional homolog of CD2. The term includes, then, various leukocytes from humans, primates (e.g., chimpanzees), mammals (e.g. carnivores, rodents, artiodactyls, perissodactyls, etc.) and vertebrates (e.g., fish, amphibians, reptiles, birds, etc.).

Typically, an antibody or ligand attached to a solid support is used to select and remove the cells from the remainder of the culture. The solid support can be a polystyrene dish (panning) or agarose beads (immunoprecipitation or affinity chromatography) or magnetic particles. The cells that attach to the antibodies or ligands are separated from the remainder of the culture and washed to eliminate any contaminating cells. Alternatively, the cell culture is divided into cell types by their ability to bind to fluorescent dye-tagged antibodies. The subgroups of cells, after incubation with the tagged antibodies, are separated by a fluorescence activated cell sorter (FACS). In either method, the cells are released from the antibodies or ligands either by intracellular mechanisms or by removal by competition with antibody ligands or receptors with a higher binding affinity than the cell has for the antibody or ligand.

The phrase "Th0 cells" refers to $CD2^+$ lymphocytic cells which are precursors to Th1 and Th2 cells. These cells express cytokines that both Th1 and Th2 cells express. Th0 cells differentiate, as a consequence of priming, into "Th1 cells," which produce primarily IL-2, interferon and TNF-β, and "Th2 cells," which mainly produce IL-4, IL-5, IL-6 and IL-10. Th1 cells are effective inducers of cellular immune responses while Th2 cells are effective in helping B cells develop into antibody-producing cells. "Overfunctionalized Th1 cells" refers to Th1 cells that, because of induction, express greater amounts of cytokines than uninduced Th1 cells produce.

The phrase "higher percentage of Th1 cells" refers to an increase of the relative number of Th1 cells in a cell culture. This can occur through preferential differentiation of Th0 cells into Th1 cells.

The phrase "NK cells" refers to cytolytic lymphocytes responsible for the cytotoxicity observed against a variety of tumor and virus-infected cells. NK cells are probably involved in tumor resistance, host immunity to viral and other microbial infections and regulation of lymphoid and other hematopoietic cell populations.

The phrase “characterized by greater activity in the woodchuck hepatitis model” refers to compounds and compositions which are more cytotoxic in a woodchuck hepatitis model than a reference compound or composition, typically but not exclusively, a purified interferon. An exemplary woodchuck hepatitis model is described in U.S. Patent 5,246,924.

The phrase “collecting a supernatant” refers to removing a cell culture supernatant from the cell culture. Separation of the supernatant may be done by any means known to those of skill, including but not limited to, centrifugation, filtration, aspiration and decanting. The phrase “enriching a supernatant” refers to concentrating the components of a supernatant. Concentration can be by well known techniques such as, differentiation solubility, ultrafiltration, diafiltration, sedimentation velocity centrifugation, preparative electrophoresis, and molecular sieve chromatography.

The phrase “a cocktail of cytokines” refers to a plurality of different cytokines. The composition can be either solid, as in a frozen state or lyophilized, or in a liquid state, as in an aqueous solution.

The phrase “contacting a cell culture” means administering a substance to a cell culture. This is different than administering a substance to cells in that there is no requirement that the substance contact the cells per se.

The term “cytokines” refers to proteins, TNF, the interferons, interleukins, chemokines, soluble receptors and cellular growth factors produced by T-cells. The phrase “upregulating cytokines associated with the Th1 pathway” refers to inducing cells to increase expression of cytokines which are associated with the Th1 pathway, *e.g.*, IL-2 and γ-IFN. The “Th1 pathway” refers to cells along the differentiation pathway that leads to terminally differentiated Th1 cells. The phrase “downregulating cytokines associated with the Th2 pathway” refers to inducing cells to decrease expression of cytokines which are otherwise associated with the Th2 pathway, *e.g.*, IL-4, IL-5, IL-6 and IL-10. The “Th2 pathway” refers to cells along the differentiation pathway that leads to terminally differentiated Th2 cells.

The phrases “effective amount,” “amount effective to” or “pharmacologically effective amount” include reference to an amount of a tryptophan-containing compound, e.g., L-Xaa-L-Trp, sufficient to produce a desired result, such as stimulating the Th1 pathway of T-cells in an *in vitro* environment.

The term “encapsulated” refers to enclosing cells in a semipermeable pouch, matrix or membrane to separate the cells from immunoreactive cells and

molecules. Each cell can be singularly encapsulated or a culture of cells can be so encapsulated.

The phrase “equivalent amount” refers to an amount of a compound that produces an effect similar to an amount of the same or another compound. For example, an amount of a cocktail of cytokines that produces the same effect as a known amount of purified interferon would be an equivalent amount.

The phrase “healthy individual” refers to a subject, preferably a human, who is not suffering from a disease treatable by L-Glu-L-Trp or by the cocktail of cytokines produced by the contact of L-Glu-L-Trp with T-cells. The phrase “subject suffering from a pathological condition” refers to a subject, preferably a human, who is suffering from a disease treatable by L-Glu-L-Trp or by the cocktail of cytokines produced by the contact of L-Glu-L-Trp with T-cells. The term “responsive to interferon treatment” means a pathological condition is treatable by either a cocktail of interferons or by a purified single interferon. The phrase “subject does not suffer from the pathological condition” refers to a subject, preferably human, that does not suffer from a pathological condition treatable by cytokines associated with the Th1 pathway. The phrase “subject with a tumor” refers to a subject, preferably human, who has a tumor, either benign or malignant, either solid or diffused, *e.g.*, leukemia or lymphoma.

The term “immunosuppressed” refers to a pathological condition wherein the immune system does not mount an adequate response to immunological challenge. The condition can be induced by drugs, biologicals, viruses, or it can be genetically-based, *e.g.*, ADA deficiency or SCID. Immunosuppression can result from a variety of mechanisms, including but not limited to, blocking DNA synthesis, *e.g.*, treatment with methotrexate; blocking immune cell activation, *e.g.*, treatment with glucocorticoids, monoclonal antibodies and HIV infection; inducing tolerance, *e.g.*, transfusion and transplantation; and blocking antigen presentation, *e.g.*, MHC blockade.

The terms “isolated,” “purified” or “biologically pure” refer to material that is substantially or essentially free from components which normally accompany it as found in its native state. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. Because the compositions of this invention are cocktails of proteins, “purified” designates the cocktail as being substantially free of proteins added to the cell culture medium in which the cells are maintained.

The term "lyophilized" or "freeze-dried" refers to freezing and subsequent evaporation of the moisture under a strong vacuum to preserve a composition. Lyophilization can take place in individual stoppered vials in an industrial freeze-dryer under controlled conditions, or on a laboratory bench top using round bottom flasks and a vacuum pump. In addition to preservation of compositions, lyophilization also can be used to concentrate compositions by removal of water from the composition.

The phrase "membrane" refers to a thin, pliable, semipermeable matrix used as a filter, separator, *etc.* A membrane can be animal tissue in origin or it can be synthetic. A membrane should be permeable to proteins, carbohydrates and other compounds present in tissue culture fluid or in the interstitial fluid of a subject but impermeable to cells, viruses, bacteria, *etc.*

The phrase "optimal therapeutic regimen" refers to a schedule of administering a therapeutic drug of this invention, *e.g.*, L-Xaa-L-Trp, to affect the greatest therapeutic benefit. In the context of this invention, the optimal therapeutic regimen is determined by *in vitro* culturing of T-cells from a subject in need of therapy in the presence of different amounts of L-Xaa-L-Trp administered at different times. The phrase "at least once per day" refers administering or contacting L-Xaa-L-Trp at least once in a 24 hour period. "At least about 5 days" refers to administering or contacting L-Xaa-L-Trp for a period of 5 days. Therefore, if L-Xaa-L-Trp is administered once per day for 5 days, a total of 5 administrations of L-Xaa-L-Trp will be given.

The phrase "pharmaceutically acceptable carrier" refers to any of the standard pharmaceutical carriers, buffers, and excipients, such as a phosphate buffered saline solution, 5% aqueous solution of dextrose, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents and/or adjuvants. Suitable pharmaceutical carriers and formulations are described in REMINGTON'S PHARMACEUTICAL SCIENCES, 19TH ED. (Mack Publishing Co., Easton, 1995). Preferred pharmaceutical carriers depend upon the intended mode of administration of the active agent.

The phrase "plurality of differing amounts" referring to amounts in a plurality of compositions, means differing total amounts of a compound, *i.e.*, L-Xaa-L-Trp in the compositions.

"Plurality" means more than one.

The phrase "solid tumor" refers to an uncontrolled, abnormal, circumscribed growth of cells. The phrase "disseminated tumor" refers to an

uncontrolled, abnormal, noncircumscribed growth of cells. The phrase "inhibition of growth of tumor cells" refers to controlling the growth of solid tumor cells, either by killing the tumor cells or by slowing down the rate of division of the tumor cells. The phrase "hematopoietic in origin" refers to cells which are derived from the bone marrow. Hematopoietic cells differentiate into blood and lymphatic cells.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides methods for upregulating production of cytokines associated with the Th_1 pathway, and downregulating production of cytokines associated with the Th_2 pathway. The methods involve *ex vivo* treatment of $CD2^+$ cells, either in a primary cell culture or an immortalized cell line, with tryptophan-containing compounds. These compounds increase the production of certain cytokines from those cells, including the interferons and IL-2, and decrease production of other cytokines, including IL-4, IL-5, IL-6 and IL-10. The cocktail of cytokines produced by the cells can then be used to treat a plethora of diseases responsive to cytokine treatment. In particular, the cocktail is useful to treat diseases currently treated by individual cytokines. In addition to pharmaceutical applications, the cocktails of this invention are useful in *in vitro* applications, *e.g.*, a provider of cytokines and growth factors necessary for the maintenance of certain cells in tissue culture.

I. TRYPTOPHAN-CONTAINING COMPOUNDS

Tryptophan-containing compounds are useful in the methods of this invention for producing cytokines, including but not limited to, IL-2, α-IFN and γ-IFN. These tryptophan-containing compounds have the formulae:

(1) L-Xaa-L-Trp, wherein Xaa is Glu, Ile or Leu;

(2) a cyclic form of L-Xaa-L-Trp;

(3) a linear or cyclic polymer of L-Xaa-L-Trp, the polymer having no more than 20 or no more than 10 amino acids; and

(4) derivatives of any of the foregoing compounds which hydrolyze in aqueous solution into any of the foregoing compounds.

Regarding cyclized forms of the compound, it is well known in the art of chemistry that peptides frequently exist in solution in equilibrium between linear and cyclized states, equilibrium favoring the linear state. Therefore, in the blood, cyclic L-Xaa-L-Trp would tend to equilibrate into the linear form. Regarding linear or cyclic

polymers of L-Xaa-L-Trp, once introduced into the body, these compounds undergo proteolytic degradation, thereby releasing the most active form of the compound, L-Xaa-L-Trp.

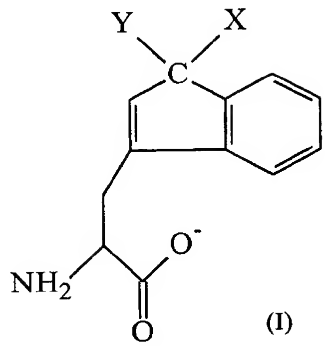
Derivatives of L-Xaa-L-Trp also are useful in the treatments of this invention. In one embodiment, a derivative is a pharmaceutically acceptable salt of the above compounds. A "pharmaceutically acceptable salt" is a salt that can be formulated into a compound for pharmaceutical use including, *e.g.*, metal salts (sodium, potassium, magnesium, calcium, *etc.*) and salts of ammonia or organic amines.

In another embodiment, a derivative is an analog in which the reactive terminal amine or carboxyl groups are derivatized with amides, imides, esters, anhydrides, ethers, methyl or ethyl-alkyl esters, alkyl, aryl or mixed alkyl/aryl moieties in which the formula weight of the entire compound is less than about 5000 Daltons or less than 1000 Daltons. Such derivatives are expected to equilibrate into the active form by, for example, hydrolysis in the body.

In another embodiment, the derivative is an analog in which L-amino acids are substituted for D-amino acids, such as L-Xaa-D-Trp or D-Xaa-L-Trp.

In another embodiment, a derivative is a pro-drug that is metabolized into the active form, *e.g.*, L-Xaa-L-Trp. One important class of analogs are trimeric compounds of the formula. Naa-L-Xaa-L-Trp, wherein Naa is any L-amino acid or a mono-saccharide. Naa-L-Xaa-L-Trp also is degraded upon administration to the body by the cleavage of the amino-terminal moiety into the active L-Xaa-L-Trp. Another class of analogs are those in which the compound is derivatized by the covalent attachment of a moiety at the amino or carboxy terminus. Representative examples include HEW, EWEW, GEW, EWKHG, EWKKHG, EW-NH-NH-GHK-NH2, Ac-L-Glu-L-Trp-OH, Suc-EW, Cpr-EW, But-EW, RKEWY, RKEW, KEWY, KEW, pEW.

In another embodiment the derivative is an analog of tryptophan such as 5-hydroxy-tryptamine, 5-hydroxy-indol-acetic acid or pyrole analogs in which the nitrogen in the pyrole ring is replaced with carbon. For example, the compound can have the formula L-Xaa-L-Trp*, wherein Xaa is Glu, Ile or Leu, and Trp* has the formula:



(I)

wherein X and Y are independently selected from H, lower alkyls, esters, amides, halides, carbohydrates or oligodideoxyribose groups, or, together can be a ketone group. The bond between X or Y and the carbon can be non-hydrolyzable. In that case, X or Y have a mass of less than about 500 D, preferably less than about 100 D. The bond between X or Y and the carbon can be hydrolyzable. In that case, any derivitization will result in an analog compound that, when exposed to water or to an enzyme that breaks a hydrolyzable bond, will convert or transform to a hydrate or ketone. Such forms are active. These Trp analogs, such as Trp*, can replace Trp in other derivatives mentioned here, as well.

II. $CD2^+$ CELLS

We have discovered that L-Xaa-L-Trp, when contacted to $CD2^+$ cells, drives the $CD2^+$ cells down the Th1 pathway. In traveling down that pathway, and as fully differentiated Th1 cells, $CD2^+$ cells synthesize and release characteristic cytokines which have therapeutic properties.

A. *Sources of $CD2^+$ Cells*

An aspect of this invention is the contacting of $CD2^+$ cells with tryptophan-containing compounds *in vitro*. Therefore, $CD2^+$ cells first must be removed from subjects, with or without a pathological condition. $CD2^+$ cells can be obtained in a variety of ways, some of which are described below, and some of which will become apparent to those of skill upon review of this disclosure. CD2 is found on all human peripheral T lymphocytes, thymocytes, 9% to 12% of bone marrow cells, and the majority of CD3- NK cells (FUNDAMENTAL IMMUNOLOGY, 3RD ED., Paul, (ed.), Raven Press, New York (1993)). The most ready supply of non-immortalized $CD2^+$ cells is bone marrow and peripheral blood.

Typically, one of two methods is used to remove cells from the red marrow of the bones of the spine, sternum, ribs, clavicle, scapula, pelvis and skull. To aspirate cells of the bone marrow, under spinal or general anesthetic, a needle is inserted into the marrow of the iliac crest of a pelvic bone, pelvic bones being the most accessible to biopsy. Another common site for aspiration biopsy is the sternum. An alternative method used to remove bone marrow is a trephine biopsy. In a trephine biopsy, a bone core containing red marrow is removed, again, usually from the iliac crest of the pelvis. The marrow then is separated from the bone before culturing the cells of the marrow.

After retrieval of the marrow from the patient and removal of the bone from the sample, if necessary, the mononuclear cells are isolated by density centrifugation. For example, bone marrow first is diluted in tissue culture medium supplemented with fetal bovine serum. The cells are then layered on top of a sample of Ficoll-Hypaque® (Amersham Pharmacia Biotech) in a centrifuge tube. The cells are centrifuged through the Ficoll-Hypaque for a suitable length of time, typically 30-45 minutes. After centrifugation, the mononuclear cells will appear as a white band in the Ficoll-Hypaque. The red cells will be in the bottom of the tube and the plasma with the platelets remain on top of the Ficoll-Hypaque®. The mononuclear cells are removed from the Ficoll-Hypaque® and tested for viability by techniques well known in the art, such as Trypan Blue exclusion dye. Optionally, the mononuclear cell sample is washed in tissue culture medium or phosphate buffered saline (PBS) to remove residual Ficoll-Hypaque®. The mononuclear cell population is then seeded in tissue culture flasks or sample wells at an appropriate concentration, preferably from 1×10^6 to 1×10^8/mL.

Alternatively, $CD2^+$ cells are removed from the peripheral blood. Typically, peripheral blood is removed from a subject by venipuncture. However, depending on the subject, other methods may be used, *e.g.*, cardiopuncture. Depending on the procedure used and the subject, from about 20 mL to approximately 500 mL of blood safely can be removed from a human subject. Less blood is typically taken from pediatric and geriatric subjects.

The blood should be directly drawn into a sterile container, such as a Vacutainer® tube or a blood bag. To prevent the lymphocytes from being trapped in a blood clot, the blood should be drawn into a tube which contains a clotting inhibitor, *e.g.*, heparin, citrate or other anti-clotting agents known to those of skill. White blood cells are isolated from the blood sample by techniques similar to those described above.

In an optional and preferable embodiment, the bone marrow or peripheral blood cell samples are enriched for $CD2^+$ cells. Enriching a cell population for cells which bear a specific cell surface antigen or selecting for such cells are well known in the art and include, but not limited to, rosetting, panning, FACSorting, *etc.*

Typically, an antibody directed against CD2 or the LFA-3 ligand (which binds specifically to CD2) attached to a solid support is used to select and remove the T-cells from the remainder of the cell population. The solid support can be a polystyrene dish (panning), agarose beads (immunoprecipitation or affinity chromatography) or magnetic particles. The cells that attach to the antibodies or ligands are separated from the remainder of the population and washed to eliminate any contaminating cells.

In another aspect, the bone marrow or peripheral blood cell population is enriched for $CD2^+$ cells by their ability to bind to fluorescent dye-tagged antibodies. The white blood cell population, after incubation with the tagged antibodies, is divided into $CD2^+$ and CD2- cell populations by a fluorescence activated cell sorter (FACS). In either method, the cells are released from the antibodies or ligands either by intracellular mechanisms or by competitive removal with anti-CD2 antibody ligands or LFA-3.

An alternative or additional method of enriching T-cells in a white blood cell population is by E-rosetting. T-cells are unique in that they bind, without prior sensitization or activation, to erythrocytes. Typically sheep erythrocytes are used, but rabbit and erythrocytes of other species may be used. The erythrocytes are incubated with the white blood cell population for a suitable time to allow for specific binding. Typically, approximately 2.5 x 10^6/mL cells are incubated with about 0.5-1% suspension (total) of sheep erythrocytes at 37°C for 10 min. If it is only necessary to count the T-cells, *e.g.*, not recover them, the cells which are surrounded with erythrocytes are counted. Otherwise, to remove the erythrocytes, as for enriching for $CD2^+$ cells, the cocktail of cells is separated by density gradient centrifugation as described above. Since the T-cells are bound to erythrocytes, they centrifuge to the bottom of the tube with the erythrocytes. The remaining white blood cells are aspirated with the Ficoll-Hypaque®. The erythrocytes are then lysed by a rapid incubation in a hypotonic solution. The remaining T-cells are separated from the lysed erythrocytes by centrifugation and washed.

An alternate source of $CD2^+$ cells is an immortalized cell line. Because a T-cell line consists only of $CD2^+$ cells, it is not necessary to isolate and/or enrich the culture for $CD2^+$ cells prior to use. An exemplary cell line is Jurkat, clone E6-1, available from the ATCC (ATCC TIB 152). Typically, immortal cell lines are either derived from

a tumor cell or are immortalized by infection with an oncogenic virus. Because of the tumorigenic potential of these cell lines, care must be taken when recovering the supernatant containing the cocktail of cytokines to eliminate potential tumorigenic agents also present in the cell supernatant. Methods of doing this are known in the art and include but are not limited to, downstream purification steps, pasteurization, and treatment with antiviral agents, such as phenanthroline (see, U.S. Patent 4,720,385).

B. *Characterization of $CD2^+$ Cells*

After collecting white blood cells, the cell population should be assayed for the presence of $CD2^+$ cells to ensure that most, if not all, of the cells present in the population bear CD2 on their cell surfaces. The enriched $CD2^+$ cell population can be characterized by E-rosetting or FACSorting as described above or other techniques known to those of skill may be used. These techniques include but are not limited to functional and immunochemical assays.

Functional assays determine the presence of $CD2^+$ cells by detecting activity unique to CD2-bearing cells. For example, mitogenic lectins, such as phytohemagglutinin (PHA), induce Ca^{2+} flux in T-cells. This flux is blocked by anti-CD2 antibodies. Thus, the change in Ca^{2+} across the membrane of a suspect $CD2^+$ cell in the presence of PHA is a functional assay for $CD2^+$ cells.

Similarly, proliferation of cells in the presence of antibodies directed against anti-CD2 antibodies can be used to determine the relative quantity of $CD2^+$ cells in a population. Proliferation of T-cells is induced by any of a variety of known T-cell stimulators, *e.g.*, phorbol 12-myristate 13-acetate (PMA). Stimulation of T-cells causes the cells to increase expression of certain proteins, *e.g.*, IL-2, as well as proliferation of the cells. This proliferative effect is mediated by CD2. Thus, if cells in a population express CD2, the addition of antibodies directed against CD2 would inhibit the proliferation of the PMA-stimulated cells.

1. **Immunochemical Binding Assays.**

Immunochemical assays are based on specific binding between a target analyte and an antibody. Specific binding requires an antibody that is selected for its specificity for a particular analyte. $CD2^+$ cells, with or without stimulation, express a number of proteins and other compounds which can be detected in the cell culture medium, *e.g.*, IL-2, IL-3, IL-4, IL-5, interleukin 10, γ-IFN, granulocyte-macrophage

colony stimulating factor (GM-CSF), TNFα and TNFβ. These compounds can be used as analytes which are recognized and bound specifically by antibodies in an immunochemical assay.

In a preferred embodiment, compounds expressed by $CD2^+$ cells into the culture medium are detected and/or quantified using any of a number of well recognized immunochemical binding assays (see, *e.g.*, U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168). For a review of the general immunoassays, see also METHODS IN CELL BIOLOGY Vol. 37: *Antibodies in Cell Biology*, Asai, ed. Academic Press, Inc. New York (1993); and Stites. Immunochemical binding assays (or immunoassays) typically utilize a "capture agent" to specifically bind to and often immobilize the analyte (in this case compounds from $CD2^+$ cells or cytokines). The capture agent is a moiety that specifically binds to the analyte. In a preferred embodiment, the capture agent is an antibody. The antibody (*e.g.*, anti-TNFβ) may be produced by any of a number of means well known to those of skill in the art and as described above.

Immunoassays also often utilize a labeling agent to specifically bind to and label the binding complex formed by the capture agent and the analyte. The labeling agent may itself be one of the moieties comprising the antibody/analyte complex. Thus, the labeling agent may be a labeled polypeptide or a labeled antibody. Alternatively, the labeling agent may be a third moiety, such as another antibody, that specifically binds to the antibody-analyte complex.

In a preferred embodiment, the labeling agent is a second antibody bearing a label. Alternatively, the second antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second can be modified with a detectable moiety, such as biotin, to which a third labeled molecule can specifically bind, such as enzyme-labeled streptavadin.

Other proteins capable of specifically binding immunoglobulin constant regions, such as protein A or protein G may also be used as the label agent. These proteins are normal constituents of the cell walls of streptococcal bacteria. They exhibit a strong non-immunogenic reactivity with immunoglobulin constant regions from a variety of species (*see*, generally Kronval, *et al.*, *J. Immunol.* **111**:1401-1406 (1973), and Akerstrom, *et al.*, *J. Immunol.* **135**:2589-2542 (1985)).

The particular label or detectable group used in immunoassays is not a critical aspect of the invention, so long as it does not significantly interfere with the

specific binding of the antibody used in the assay. A label can be any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels include magnetic beads (*e.g.* Dynabeads™), fluorescent dyes (*e.g.*, fluorescein isothiocyanate, Texas red, rhodamine, and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.* polystyrene, polypropylene, latex, *etc.*) beads. Detection of the label depends on the label used and will be apparent to those of skill.

Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, analyte, volume of solution, concentrations, and the like. Usually, the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 10°C to 40°C.

a. Non-Competitive Assay Formats.

Immunoassays for detecting analytes may be either competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of captured analyte (in this case a cytokine or a $CD2^{+}$ cell-related compound) is directly measured. In one preferred "sandwich" assay, for example, the capture agent (antibodies) can be bound directly to a solid substrate where they are immobilized. These immobilized antibodies then capture an analyte present in the test sample. The analytes thus immobilized are then bound by a labeling agent, such as a second antibody bearing a label. Alternatively, the second antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second can be modified with a detectable moiety, such as biotin, to which a third labeled molecule can specifically bind, such as enzyme-labeled streptavadin.

b. Competitive Assay Formats.

In competitive assays, the amount of analyte (cytokines or $CD2^{+}$ cell-related compounds) present in the sample is measured indirectly by measuring the amount

of an added (exogenous) analyte displaced (or competed away) from a capture agent (antibody) by the analyte present in the sample. In one competitive assay, a known amount of, in this case, a $CD2^+$ cell-related compound is added to the sample and the sample is then contacted with a capture agent, in this case, an antibody that specifically binds to the marker. The amount of analyte bound to the antibody is inversely proportional to the concentration of analytes present in the sample.

In a particularly preferred embodiment, the antibody is immobilized on a solid substrate. The amount of analytes bound to the antibody may be determined either by measuring the amount of analytes present in an analyte/antibody complex, or alternatively by measuring the amount of remaining uncomplexed analyte. The amount of analytes may be detected by providing a labeled analyte.

A hapten inhibition assay is another preferred competitive assay. In this assay a known analyte, in this case, a $CD2^+$ cell-related compound is immobilized on a solid substrate. A known amount of anti-marker antibody is added to the sample, and the sample is then contacted with the immobilized marker. In this case, the amount of antibody bound to the immobilized marker is inversely proportional to the amount of marker present in the sample. Again the amount of immobilized antibody may be detected by detecting either the immobilized fraction of antibody or the fraction of the antibody that remains in solution. Detection may be direct where the antibody is labeled or indirect by the subsequent addition of a labeled moiety that specifically binds to the antibody as described above.

c. Other Assay Formats.

In another preferred embodiment, Western blot (immunoblot) analysis is used to detect and quantify the presence of particular $CD2^+$ cell-related compounds in the tissue culture fluid. The technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter) and incubating the sample with antibodies that specifically bind the $CD2^+$ cell-related compounds. The antibodies specifically bind to markers on the solid support. These antibodies may be directly labeled or alternatively may be subsequently detected using labeled antibodies (*e.g.*, labeled sheep anti-mouse antibodies) that specifically bind to the markers.

Other assay formats include liposome immunoassays (LIA), which use liposomes designed to bind specific molecules (*e.g.*, antibodies) and release encapsulated reagents or markers. The released chemicals are then detected according to standard techniques (*see*, Monroe *et al.*, *Amer. Clin. Prod. Rev.* **5**:34-41 (1986)).

Those who practice in the art of immunoassays understand that other factors need to be considered when optimizing immunoassays. For example, non-specific binding of the assay components should be minimized by using buffers containing protein compositions such as bovine serum albumin (BSA), nonfat powdered milk, and gelatin.

2. Antibodies

Antibodies are used to bind CD2$^+$ cells in the immunochemical assays described above. Methods of producing polyclonal and monoclonal antibodies are known to those of skill in the art. *See, e.g.*, Coligan, CURRENT PROTOCOLS IN IMMUNOLOGY, Wiley/Greene, NY (1991); Stites *et al.* (eds.) BASIC AND CLINICAL IMMUNOLOGY (7th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein ("Stites"); Goding, MONOCLONAL ANTIBODIES: PRINCIPLES AND PRACTICE (2d ed.) Academic Press, New York, NY (1986); Kohler and Milstein, *Nature* **256**:495-497 (1975) ("Kohler and Milstein"); and Harlow and Lane. Such techniques include antibody preparation by selection from libraries of recombinant antibodies in phage or similar vectors. *See*, Huse *et al.*, *Science* **246**:1275-1281 (1989) ("Huse"); and Ward *et al.*, *Nature* **341**:544-546 (1989).

Production of polyclonal antibodies is well documented. In brief, an immunogen is mixed with an adjuvant and animals are immunized. Test bleeds are monitored to determine the titer of reactivity to the immunogen. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera prepared. Further fractionation of the antisera to enrich for antibodies reactive to the protein is done if desired. (See Harlow and Lane, *supra*).

Monoclonal antibodies for use in immunoassays or immunoaffinity purification may be obtained by various techniques familiar to those skilled in the art. Briefly, an animal, typically a mouse, is immunized with an immunogen, *e.g.*, TNFβ. The animal is sacrificed and the spleen removed. The spleen cells are immortalized, commonly by fusion with myeloma cells (*See*, Kohler and Milstein, *Eur. J. Immunol.* **6**:511-519 (1976)). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, retroviruses, or other methods well known in the art.

Colonies of clonal cells arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the analyte.

Alternatively, one may isolate DNA sequences which encode a monoclonal antibody or a binding fragment thereof by screening a DNA library from human B cells according to the general protocol outlined in Huse. The monoclonal antibodies produced by the above methods are then pooled to create a cocktail which specifically binds to analytes of interest.

C. *Encapsulation*

If desired, the $CD2^+$ cells of this invention can be implanted into a subject. This is a preferable treatment for immune suppressed individuals or others who are incapable of synthesizing the cytokines of this invention. If the implant is to be allogeneic, the cells must first be encapsulated.

Encapsulation of cells for implants in *in vivo* diabetic models has been reported by several research groups. *See*, Lum *et al.*, *Diabetes* **40**:1511 (1991); Maki *et al.*, *Transplantation* **51**:43 (1991); Scharp *et al.*, *Diabetes* **39**:515 (1990); Robertson, *Diabetes* **40**:1085 (1991); Colton *et al.*, *J. Biomech. Eng.* **113**:152 (1991); Reach, *Intern. J. Art. Organs* **13**:329 (1990); and Warnock *et al.*, *Diabetes* **37**:467 (1988). *See also*, U.S. Pat. No. 3,827,565; U.S. Pat. No. 4,323,457; U.S. Pat. No. 4,242,459; U.S. Pat No. 4,242,460; U.S. Pat. No. 4,391,909; U.S. Pat. No. 4,378,016; U.S. Patent No. 5,427,940; U.S. Patent No. 5,262,055.

The cells may be encapsulated by membranes prior to implantation. The encapsulation provides a barrier to the host's immune system and inhibits graft rejection and inflammation. Several methods of cell encapsulation may be employed. In some instances, cells will be individually encapsulated. In other instances, many cells will be encapsulated within the same membrane. If necessary to remove the cells, the relatively large size of a structure encapsulating many cells within a single membrane provides a convenient means for retrieval of the implanted cells. Several methods of cell encapsulation are well known in the art, such as described in European Patent Publication No. 301,777, or U.S. Patent Nos. 4,353,888, 4,744,933, 4,749,620, 4,814,274, 5,084,350, or 5,089,272.

One method of cell encapsulation is as follows. The $CD2^+$ cells are mixed with sodium alginate (a polyanionic seaweed extract) and extruded into calcium chloride so as to form gel beads or droplets. The gel beads are incubated with a high molecular

weight (60-500 x 10^3 daltons) concentration (0.03-0.1% w/v) polyamino acid, such as poly-L-lysine, for a brief period of time (3-20 minutes) to form a membrane. The interior of the formed capsule is reliquified by treating with sodium citrate. The single membrane around the cells is highly permeable (MW cut-off 200-400 x 10^3 daltons). The single membrane capsule containing the cell is incubated in a saline solution for 1-3 hours to allow entrapped sodium alginate to diffuse out of the capsule and expand the capsule to an equilibrium state. The resulting alginate-poor capsule is reacted with a low molecular weight polyamino acid (10-30 x 10^3 daltons) such a poly-L-lysine (PLL) or chitosan (deacetylated chitin; 240 x 10^3 daltons) to produce an interacted, less permeable membrane (MW cut-off 40-80 x 10^3 daltons). The dual membrane encapsulated cells are then cultured in suitable tissue culture medium, for example, Dulbecco's Modified Eagle's Medium for two to three weeks.

While reference has been made specifically to sodium alginate beads, it will be appreciated by those skilled in the art that any non-toxic water soluble substance that can be gelled to form a shape-retaining mass by a change in conditions in the medium in which it is placed may be employed. Such gelling material generally comprises several chemical moieties which are readily ionized to form anionic or cationic groups so that the surface layers can cross link to form a permanent membrane when exposed to oppositely charged polymers. Most polysaccharide gums, both natural and synthetic, can be cross-linked by polymers containing positively charged reactive groups such as amino groups. The cross-linking biocompatible polymers which may be reacted with the sodium alginate gum include polylysine and other polyamino acids. The degree of permeability of the membrane formed may be controlled by careful selection of a polyamino acid having the desired molecular weight. PLL is the preferred polymeric material but others include chitosan and polyacrylate. Molecular weights typically vary from about 10^4 to about 10^6.

III. CONTACTING CD2$^+$ CELLS WITH TRYPTOPHAN-CONTAINING COMPOUNDS

To produce the cocktail of cytokines of this invention, CD2$^+$ cells are contacted with tryptophan-containing compounds, more preferably, L-Xaa-L-Trp. The L-Xaa-L-Trp can be added to the culture of CD2$^+$ cells as a solid, *e.g.*, lyophilized or crystallized, or in a solution. The amount of L-Xaa-L-Trp necessary to produce the cocktail of cytokines ranges from about 1 x 10^6 to about 4 x 10^6 molecules per CD2$^+$ cell in culture, more preferably from about 2 x 10^6 molecules to 4 x 10^6 molecules per CD2$^+$

cell. The most preferable amount of L-Xaa-L-Trp to be contacted is 1×10^6 to 4×10^6 molecules per $CD2^+$ cell. However, any amount effective to induce T-cells to increase production of cytokines associated with the Th1 pathway, including the interferons, can be used.

Because $CD2^+$ cells do not become refractory to L-Xaa-L-Trp, the dipeptide can be added at any time for any number of times to produce the cocktail of this invention. For continuous production of the cocktail of cytokines, *e.g.*, from an immortalized cell line, the contacting of L-Xaa-L-Trp can be continuous. For example, the continuous presence of L-Xaa-L-Trp in a cell culture maintained in a continuous perfusion bioreactor.

One of skill will realize that the number of $CD2^+$ cells used to produce the cocktail of cytokines will depend on the container used, *e.g.*, 96 well plates, tissue culture flasks, spinner flasks, or large production scale fermenters. Similarly, the concentration of $CD2^+$ cells in the container will depend on the culture conditions, *e.g.*, gaseous exchange, media content, agitation of suspended cultures, surface area of adherent cultures, *etc.* However, as a rule, the concentration of $CD2^+$ cells should be on the order of about 10^5 - 10^8 cells/mL.

IV. COCKTAIL OF CYTOKINES

Upon contact with the tryptophan-containing compounds of this invention, $CD2^+$ cells are driven along the Th1 pathway and overexpress certain cytokines and underexpress others. For example, after exposure to L-Glu-L-Trp, $CD2^+$ cells overexpress IL-2, interferons, and other cytokines associated with cell-mediated immunity. $CD2^+$ cells along the Th1 pathway underexpress IL-4, IL-5, IL-6, and IL-10. Because of variability in the $CD2^+$ cells across a population of individuals, the relative amount of each cytokine in the cocktails will depend on the source of the cells. Accordingly, in another aspect, this invention provides cocktails of cytokines produced by $CD2^+$ cells driven along the Th_1 pathway.

The therapeutic compositions of this invention comprise a cocktail of cytokines which, compared with compared with a cocktail produced by cultured, untreated peripheral white blood cells, is characterized by a statistically significant increase (e.g., $p < 0.05$ or $p < 0.01$) in the amount of at least one, preferably a plurality, of cytokines associated with the Th1 pathway and a statistically significant decrease (e.g., $p < 0.05$ or $p < 0.01$) in at least one, preferably a plurality, of cytokines associated with the

Th2 pathway. In certain embodiments, the amount of the Th1-associated cytokine can be at least 20%, at least 50% or at least 100% greater than in that produced by cultured, untreated blood cells. The amount of the Th2-associated cytokine can be less than 95%, less than 90%, less than 80% or less than 50% that of cultured, untreated cells. The therapeutic compositions of this invention also can be characterized as being purified, i.e., separated from naturally occurring serum components, such as albumin. The therapeutic compositions also can be enriched, e.g., can comprise a significantly increased concentration of the cytokines compared with normal serum.

In one aspect of this invention, $CD2^+$ cells are chosen so as to "tailor" the cocktail of cytokines for the subject to be treated. In one embodiment, the $CD2^+$ cells from the subject with a pathological condition are contacted with the tryptophan-containing compounds of this invention to produce a cocktail that is optimum for that subject. In another embodiment, the $CD2^+$ cells are from a subject who does not have the pathological condition to produce a cocktail that is optimum against the pathological condition. In yet another embodiment, the $CD2^+$ cells are an immortalized cell line. These cells produce a cocktail that is constant from batch to batch and can be validated for commercial pharmaceutical use.

A. *Sources of Cytokines*

After contacting the $CD2^+$ cells with the tryptophan-containing compounds of this invention, the cell supernatant is collected. Those of skill in the art will be aware that many different methods can be used to collect cell supernatants; three preferred methods being settling, centrifugation and filtration. In a most preferred embodiment, all three are utilized to remove cells and cellular debris from the cell supernatant.

1. Settling

Settling is the use of gravity to allow suspended cells, *e.g.*, T-cells, to settle in the bottom of the container. The supernatant is then decanted from the remaining cells. This technique can be used in a range of formats; from wells of a microtiter plate and test tubes for small volumes, to settling devices in large bioreactors. If the cells are in wells of a microtiter plate, the cells rest at the bottom of the well (unless there is some sort of stirring device within the well) and the supernatant is removed by drawing it up in a pipet. If the cells are in a test tube, tissue culture or spinner flask, the cells are allowed to drop

to the bottom of the tube or flask and the supernatant is decanted from the cells by pouring. Large bioreactors have settling chambers which allow the cells to fall to the bottom of the chamber and be returned to the bioreactor. The supernatant is continuously drawn from the top of the settling chamber. For examples of bioreactor settling devices, see, U.S. Patent Nos. 4,748,123; 5,320,963 and 5,342,781. In a batch-style fermenter, the cells and the supernatant are removed from the fermenter and the cells are allowed to settle in a separate container.

However, even the most efficient settling device will not remove all of the cells and cellular debris in a cell supernatant. Thus, in a more preferred embodiment, settling is followed by centrifugation.

2. Centrifugation

Centrifugation techniques are well known in the art and will not be described in detail. Typically, for mammalian cell cultures, suitable centrifugation to remove cells from a supernatant is 800 x g for 10-15 minutes, optimally under refrigeration. The supernatant can either be decanted from the cell pellet or removed from the cell pellet with a pipet. To prevent T-cell debris and cellular enzymes from contaminating the supernatant, the cell sample should not be centrifuged at a speed sufficient to break the cells.

Centrifugation at 800 x g removes cells and large pieces of cellular debris, but small organelles and fragments of membranes may remain in the supernatant. Thus, in a most preferred embodiment, the preceding two steps are followed by sterile filtration.

3. Filtration

Again, filtration techniques are well known in the art and will not be addressed herein. It is not preferred to use filtration as the only step or the first step in the removal of cells from cell supernatant in that large numbers of cells tend to clog the filtration membranes. Therefore, sterile filtration through a 0.44 μM or a 0.22 μM filter should be performed only after preliminary cell removal steps, as described above, have been undertaken.

B. *Detection and Characterization of Cytokines*

After the cells have been removed, it is desirable but not necessary to characterize the cocktail for cytokine content. Suitable characterization assays include,

but are not limited to fluid or gel precipitin reactions, agglutination assays, immunodiffusion (single or double), immunoelectrophoresis, immunosorbent assays, various solid phase assays, immunochromatography (*e.g.*, lateral flow immunochromatography) and more preferably radioimmunoassay, enzymatic immunoassay and most preferably ELISA. Methods of performing such assays are well known to those of skill in the art (see, *e.g.*, U.S. Patents 4,366,241; 4,376,110; 4,517,288; 4,837,168; 5,405,784; 5,534,441; 5,500,187; 5,489,537; 5,413,913; 5,209,904; 5,188,968; 4,921,787; and 5,120,643; British Patent GB 2204398A; European patent EP 0323605 B1; METHODS IN CELL BIOLOGY VOLUME 37: ANTIBODIES IN CELL BIOLOGY, Asai, ed. Academic Press, Inc. New York (1993); AND BASIC AND CLINICAL IMMUNOLOGY 7TH EDITION, Stites & Terr, eds. (1991)).

Alternatively, a functional assay can be used to detect and characterize the cocktail of cytokines. The cytokines associated with the Th1 pathway cause proliferation of many different cell types, including cytotoxic T-cells, macrophages and monocytes. Therefore, proliferation of these cells can be used as a functional assay for the cocktail of this invention. For example, CTLL-2 is an IL-2-dependent murine cytotoxic T-cell line. A proliferation assay for IL-2 using this cell line is reported in Gearing & Bird, LYMPHOKINES AND INTERFERONS, A PRACTICAL APPROACH, Clemens, *et al.*, (eds.), IRL Press (1987).

Interferon activity present in the cocktail can be measured by its anti-viral activity. In one assay, L-929 murine cells infected with EMC virus is grown with and without the cocktail. The relative rates of cell culture proliferation are determined to quantify the amount of interferon-like activity that is present in the cocktail (*see*, Vogel, *et al.*, *Infect. Immunol.* **38**:681 (1982)). In another, *in vivo* assay, woodchucks (Marmota monax) are infected with woodchuck hepatitis virus (WHV).

Like humans, woodchucks can be chronically infected with a hepadnavirus, in this case, WHV. As a result, woodchucks have become a model treatment for anti-HBV therapies as well as antiviral therapy, in general. The animals can be infected with WHV at birth or at any time prior to characterization of the cocktail of this invention. Infection of woodchucks with WHV is described in Hantz, *et al.*, *J. Virol. Methods* **7**:45 (1983).

The cocktail of this invention is administered to the WHV-infected woodchucks along with a control non-infected animal. The amount of the cocktail given to the animal depends on the relative concentration of the individual cytokines present.

However, typically an equivalent amount of γ-IFN is given to the animal. The γ-IFN in the cocktail is determined by ELISA or another method mentioned above. After determining the dose of purified γ-IFN necessary to achieve a therapeutically beneficial affect, the same dose of cocktail is given. In a preferred embodiment, the cocktail is greater than 25% more active than an equivalent amount of γ-IFN. In a most preferred embodiment, the cocktail is greater than 50% than an equivalent dose of γ-IFN. For a positive control, at least one infected woodchuck is given a placebo treatment (typically, normal saline). The cocktail can be administered by any means desirable with the preferred administration by intraperitoneal injection. Alternatively, the cocktail can be administered intravenously. The cocktail can be administered as often as desired, with twice daily preferred and once daily most preferred.

The woodchuck hepatitis assay is ultimately scored by the presence or absence of hepatocellular carcinoma. However, a more rapid assay can be performed. It requires titering the virus present in the treated versus placebo control animals. Virus titers can be done by *in vitro* plaque assays, which are well known in the art, as well as detection of viral sequences by PCR amplification and gel electrophoresis, again, techniques well known in the art. For a more detailed discussion of detection of WHV viral sequences see, U.S. Patent 5,246,924 and the references cited therein.

C. *Purification of Cytokines.*

The cocktail of cytokines of this invention may be purified to substantial purity by standard techniques, including selective precipitation with such substances as ammonium sulfate; column chromatography, immunopurification methods, and others. *See*, for instance, R. Scopes, PROTEIN PURIFICATION: PRINCIPLES AND PRACTICE, Springer-Verlag: New York (1982), U.S. Patent No. 4,673,641, Ausubel, and Sambrook, all incorporated herein by reference.

1. Solubility Fractionation

Often as an initial step and if the protein mixture is complex, an initial salt fractionation can separate many of the unwanted host T-cell proteins (or proteins derived from the cell culture media) from the recombinant protein of interest. The preferred salt is ammonium sulfate. Ammonium sulfate precipitates proteins by effectively reducing the amount of water in the protein mixture. Proteins then precipitate on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at lower

ammonium sulfate concentrations. A typical protocol is to add saturated ammonium sulfate to a protein solution so that the resultant ammonium sulfate concentration is between 20-30%. This will precipitate the most hydrophobic of proteins. The precipitate is discarded (unless the protein of interest is hydrophobic) and ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, either through dialysis or diafiltration. Other methods that rely on solubility of proteins, such as cold ethanol precipitation, are well known to those of skill in the art and can be used to fractionate complex protein mixtures.

2. Size Differential Filtration

If the size of the protein of interest is known or can be estimated from the cDNA sequence, proteins of greater and lesser size can be removed by ultrafiltration through membranes of different pore size (for example, Amicon or Millipore membranes). As a first step, the protein mixture is ultrafiltered through a membrane with a pore size that has a lower molecular weight cut-off than the molecular weight of the protein of interest. The retentate of the ultrafiltration is then ultrafiltered against a membrane with a molecular cut off greater than the molecular weight of the protein of interest. The recombinant protein will pass through the membrane into the filtrate. The filtrate can then be chromatographed as described below.

3. Column Chromatography

Proteins can be separated on the basis of their size, net surface charge, hydrophobicity and affinity for ligands. In addition, antibodies raised against proteins can be conjugated to column matrices and the proteins immunopurified. All of these methods are well known in the art.

It will be apparent to one of skill that chromatographic techniques can be performed at any scale and using equipment from many different manufacturers (*e.g.*, Pharmacia Biotech).

V. ADMINISTRATION OF THE COMPOSITIONS AND CELLS OF THIS INVENTION

A. *Administration of the Cocktails of this Invention*

This invention also provides methods of treating subjects suffering from pathological conditions responsive to interferon treatment. The methods comprise administering to the subject a cocktail of cytokines of this invention. The pathological conditions, include but are not limited to, inflammation, cancer, and autoimmune disease. Inflammation is caused by the overexpression of cytokines associated with the Th2 pathway, in particular IL-6. The cocktail of cytokines of this invention can be used to suppress Th2 cells and, through the γ-IFN present in the cocktail, drive T-cells present at the site of inflammation to the Th1 pathway.

In another aspect of this embodiment related to cancer, the response from Th1 cells can be tailored to a specific tumor cell. In this embodiment, T-cells from a patient are contacted with L-Xaa-L-Trp in the presence of the patient's tumor cells. The combination of tumor cell antigens and L-Xaa-L-Trp provide an environment in which Th1 cells mount the most effective defense. The cocktail produced by the Th1 cells is then returned to the patient to attack the tumor cells.

The cocktails of this invention preferably are delivered as pharmaceutical compositions. "Pharmaceutical composition" refers to a composition suitable for pharmaceutical use in a subject. The pharmaceutical compositions of this invention comprise a pharmacologically effective amount of a compound of the invention and a pharmaceutically acceptable carrier. "Pharmaceutically acceptable carrier" refers to any of the standard pharmaceutical carriers, buffers, and excipients, such as a phosphate buffered saline solution, 5% aqueous solution of dextrose, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents and/or adjuvants. Suitable pharmaceutical carriers and formulations are described in REMINGTON'S PHARMACEUTICAL SCIENCES, 19TH ED. (Mack Publishing Co., Easton, 1995). Preferred pharmaceutical carriers depend upon the intended mode of administration of the active agent.

The compounds of the invention can be formulated for administration in a variety of ways. Typical routes of administration include both enteral and parenteral. These include, but are not limited to, subcutaneous, intramuscular, intravenous, intraperitoneal, intramedullary, intrapericardiac, intrabursal, oral, sublingual, ocular,

nasal, topical, transdermal, transmucosal, or anal. The mode of administration can be, *e.g.*, via swallowing, inhalation, injection or topical application to a surface (*e.g.*, eyes, mucus membrane, skin).

Particular formulations typically are appropriate for specific modes of administration. Various contemplated formulations include, for example, aqueous solutions, solid formulations, aerosol, gas, vapor or dry powder formulations and transdermal formulations.

The concentration of the cytokines in the formulation will vary depending on, for example, the other compositions in the formulation, the mode of administration, the concentration of cytokines in the cocktail, the relative concentrations of each individual cytokine in the cocktail, the particular culture of $CD2^+$ cells used to produce the cocktail, and the needs of the recipient of the formulation.

B. *Aqueous Solutions for Enteral, Parenteral Or Transmucosal Administration*

Examples of aqueous solutions include, for example, water, saline, phosphate buffered saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions and the like. A preferred carrier for delivery of the cocktails of cytokines of this invention is normal (0.09%) saline solution.

The compositions can contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents, wetting agents, detergents and the like. Additives can also include additional active ingredients such as bactericidal agents, or stabilizers. For example, the solution can contain sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate or triethanolamine oleate. These compositions can be sterilized by conventional, well-known sterilization techniques, or can be sterile filtered. The resulting aqueous solutions can be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration.

Aqueous solutions are appropriate for injection (*e.g.* intravenous injection). Aqueous solutions also are useful for enteral administration as tonics and administration to mucous or other membranes as, *e.g.*, nose or eye drops. The composition can contain the cocktail in an amount of about 1 μg/mL to about 10 mg/mL

protein, more preferably about 10 μg/mL to about 1 mg/mL protein, *e.g.*, about 100 μg/mL protein.

C. *Solid and Other Non-Aqueous Compositions For Enteral Or Transdermal Delivery*

Solid compositions are appropriate for enteral administration. They can be formulated in the form of, *e.g.*, pills, tablets, powders or capsules. For solid compositions, conventional solid carriers can be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed.

The carrier can be selected from various oils including those of petroleum, animal, vegetable or synthetic origin, for example, peanut oil, soybean oil, mineral oil, sesame oil, and the like. Suitable pharmaceutical excipients include starch, cellulose, talc, glucose, lactose, sucrose, gelatin, maltose, rice, flour, chalk, silica gel, magnesium stearate, sodium stearate, glycerol monostearate, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol, and the like.

A unit dosage form, such as a tablet, can have about 1 μg to about 100 mg of cocktail protein. Some action must be undertaken to protect the composition from the proteolytic action of the gastric environment. Those of skill will recognize upon review of this disclosure other formulations that will provide such protection.

D. *Topical Administration For Transdermal Or Transmucosal Delivery*

For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated can be used in the formulation. Such penetrations are generally known in the art and include, for example, for transmucosal administration, bile salts and fusidic acid derivatives. In addition, detergents can be used to facilitate permeation. Transmucosal administration can be through nasal sprays, for example, or using suppositories. Transdermal delivery systems can include, *e.g.*, patches.

For topical administration, the agents are formulated into ointments, creams, salves, powders and gels. In one embodiment, the transdermal delivery agent can be DMSO. The concentration of the proteins of the cocktail will, of course, vary

depending on the delivery system, but preferably is from about 0.5% to about 20%, most preferably at the low end of the range and most preferably from about 1 - 2% of the formulation.

E. *Delivery By Inhalation*

For inhalation, the compound is preferably administered in the form of an aerosol or mist. For aerosol administration, the compound preferably is supplied in finely divided form along with a surfactant and propellant. The concentration of the cocktail will vary depending on the pathological condition to be treated but preferably is about 10 μg/mL to about 1 mg/mL.

The surfactant preferably is soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride such as, for example, ethylene glycol, glycerol, erythritol, arabitol, mannitol, sorbitol, the hexitol anhydrides derived from sorbitol, and the polyoxyethylene and polyoxypropylene derivatives of these esters. Mixed esters, such as mixed or natural glycerides, can be employed. The surfactant can constitute 0.1%-20% by weight of the composition, preferably 0.25%-5%.

The balance of the composition is ordinarily propellant. Liquefied propellants are typically gases at ambient conditions, and are condensed under pressure. Among suitable liquefied propellants are the lower alkanes containing up to 5 carbons, such as butane and propane; and preferably fluorinated or fluorochlorinated alkanes. Mixtures of the above can also be employed. In producing the aerosol, a container equipped with a suitable valve is filled with the appropriate propellant, containing the finely divided compounds and surfactant. The ingredients are thus maintained at an elevated pressure until released by action of the valve.

A nebulizer or aerosolizer device for administering compounds of this invention typically delivers a dose of about 1 $\mu g/m^3$ to about 10 mg/m^3.

F. *Other Formulations*

In preparing pharmaceutical compositions of the present invention, it can be desirable to modify the complexes of the present invention to alter their pharmacokinetics and biodistribution. For a general discussion of pharmacokinetics, SEE,

REMINGTON'S PHARMACEUTICAL SCIENCES, *supra*, Chapters 37-39. A number of methods for altering pharmacokinetics and biodistribution are known to one of ordinary skill in the art. Examples of such methods include protection of the complexes in vesicles composed of substances such as proteins, lipids (for example, liposomes), carbohydrates, or synthetic polymers.

VI. ADMINISTRATION OF CELLS OF THIS INVENTION

This invention also provides methods of treating subjects suffering from a pathological condition responsive to cytokines. The methods comprise administering to the subject $CD2^+$ cells treated *ex vivo* with a tryptophan-containing compound of this invention. In a preferred embodiment, T-cells are removed from the subject and after contact with L-Xaa-L-Trp, the cells are re-infused into the subject. Alternatively, if the pathological condition is isolated to a readily accessible site, *e.g.*, inflammatory lesion, the differentiated Th1 cells are administered directly to the site.

Repeatedly, it has been observed that cancers run a different course in different individuals. In part, this is due to the heterogeneity of tumor cells. However, another factor is differences between the individuals with cancer. The capability of an immune system to clear foreign antigens is, in large part, dependent on the general health of the individual. Individuals with cancer tend to be in poor immunological health, but by priming an individual's own T-cells to produce the cocktail of this invention and mimic the individual's attack of a foreign antigen when that individual is healthy, a functional immune response can be restored to that individual.

In another embodiment, the cells of another individual are infused into a subject suffering from a pathological condition, including but not limited to, cancer. For example, a factor which determines the susceptibility of an individual to cancer is the genotype of the individual. Susceptibility to some breast cancers is highly inheritable. In these women, it would be advantageous to administer Th1 cells from an individual who is not susceptible to cancer to a cancer-susceptible individual, provided care is taken not to elicit an immune response directed against the Th1 cells.

In another embodiment directed to cancer therapy, Kaposi's Sarcoma and other angiogenic tumors require IL-6 for proliferation. The primed T-cells can be used as above to treat these tumors. The primed T-cells can be used to affect, among others, treatment of autoimmune diseases, restoration of immune function following ablative

therapy, congenital immunodeficiency and many which will become apparent to those of skill upon review of this disclosure.

The cells of this invention can be delivered in many different formulations. As mentioned above, if allogeneic cells are to be infused into a subject, an immune response against the allogeneic cells must be inhibited. This can be done by encapsulating the cells as described above, or by treating the subject with immunosuppressive drugs, such as methylprednisone, 2-chlorodeoxyadenosine, cyclophosphamide, methotrexate or azathioprine prior to or simultaneously with the cells.

Like the cocktails of this invention, the cells can be administered parenterally, preferably intravenously. However, one of skill will realize that some parenteral routes of administration, such as enteral and inhalation, are not appropriate for the delivery of cells to a patient. In addition, one of skill will recognize the requirements of cells necessitate physiologically compatible solutions. Preferable solutions include, but are not limited to, the physiological solutions listed above, *e.g.*, normal saline, dextrose/saline and Ringer's solution.

In addition to parenteral administration, the cells of this invention can be administered directly to a specific site, *e.g.*, a tumor. Desired sites are internal, *i.e.*, not on the skin or on an exposed surface of the body. Administration to a desired site should be via injection, either intravenously, intramuscularly, intraperitoneally, or intraarticular, depending on the location of the desired site.

VII. OPTIMAL THERAPEUTIC REGIMEN

This invention also provides methods of identifying an optimal therapeutic regimen for treating a tumor with cytokines. The methods involve co-cultivating multiple cultures of $CD2^+$ cells of the invention with tumor cells of the subject and adding to the $CD2^+$ cell cultures, a different amount of a tryptophan-containing compound of this invention at different time intervals. The amount and interval that results in the optimal tumor regression is then used as an optimal therapeutic regimen. In this embodiment, different amounts, most preferably at least 5 different amounts, of L-Xaa-L-Trp are contacted with the subject's white blood cells in the presence of the tumor *in vitro* to determine which amount elicits the most beneficial effect, *i.e.*, reduction of tumor size. The optimum amount of L-Xaa-L-Trp is that which reduces the size of the tumor, or inhibits tumor cell culture growth, about 3-fold. The amount of L-Xaa-L-Trp required to

treat the subject is then extrapolated from the optimum amount of L-Xaa-L-Trp determined from the cell cultures.

In addition to differing amounts, the L-Xaa-L-Trp can be added at different times and for differing courses of treatment to determine the optimal therapeutic regimen. In a preferred embodiment, L-Xaa-L-Trp is contacted with the cells *in vitro* at least about once per day for about 5 days.

The tumor cells can be any type of neoplasm, *i.e.*, benign or malignant. However, because treatment will be more critical for malignant tumors, this type of tumor is preferred. The tumors can be either solid or hematopoietic or of white blood cells, *e.g.*, leukemia or lymphoma.

The $CD2^+$ cells can be from any source of such cells, *e.g.*, peripheral blood, lymph nodes, but is preferably from bone marrow. The $CD2^+$ cells are isolated from the bone marrow as described above. Typically, L-Xaa-L-Trp is added to the culture of $CD2^+$ cells prior to co-incubation with the tumor cells. The $CD2^+$ cells are removed from the L-Xaa-L-Trp containing media and washed with saline, culture media, or another appropriate solution before incubation with tumor cells. This prevents L-Xaa-L-Trp from contacting the tumor cells.

Typically, the tumor cells and the $CD2^+$ cells are separated by a membrane. The membrane allows factors to diffuse from the tumor cells into the media surrounding the $CD2^+$ cells and vice versa. However, the membrane prevents contact between the tumor cells and the $CD2^+$ cells. One of skill will realize that the pore size of the membrane must be large enough to allow macromolecules, such as proteins and carbohydrates, to pass through but small enough to prevent cells and cellular debris, including but not limited to, fragmented membranes, organelles, chromosomes, *etc.*, from traversing the barrier.

Although the present invention has been described in some detail by way of illustration and example; for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

VIII. EXAMPLE

Individualized Treatment for Breast Cancer

After biopsy, the malignant cells are either used immediately or frozen for future use. The freezing medium is RPMI-1640 supplemented with 50% heat inactivated

fetal bovine serum (Δ-FBS) and 10% DMSO. Cell culture media can be any suitable commercially prepared medium, but preferably RPMI-1640 supplemented with 15% Δ-FBS, non-essential amino acids and glutamine. The medium can be buffered with HEPES if the practitioner prefers. The cells are incubated at 37°C in a humidified 5% CO_2 incubator. After determination of malignancy, but before initiation of chemo- or radiotherapy, marrow or peripheral blood is removed from the subject. $CD2^+$ cells are isolated by the methods described herein.

The recovered $CD2^+$ cells are seeded in a tissue culture or a spinner flask at about 2 x 10^6 cells/mL in the same medium described above. L-Glu-L-Trp is added to the flask at a concentration of approximately 1 μg/mL. The cells are incubated with the dipeptide for approximately 5 days with daily exchanges of medium. After 5 days, the batches of media are combined and the cocktail purified as described above. The cocktail is formulated for parenteral pharmaceutical administration and is administered intravenously to the subject with breast cancer.

Alternatively, the $CD2^+$ cells are incubated with L-Glu-L-Trp as above but only for one day. After priming the $CD2^+$ cells to enter the Th1 pathway, the cells are returned to the subject. In the above two treatments, the therapy has been tailored to mimic the subject's optimum cell-mediated immune response in confronting a pathological condition.

The present invention provides therapeutic cytokines and methods for their production. While specific examples have been provided, the above description is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted. By their citation of various references in this document Applicants do not admit that any particular reference is "prior art" to their invention.

WHAT IS CLAIMED IS:

1. A method of upregulating cytokines associated with the Th1 pathway, said cytokines comprising interleukin 2, α-interferon and γ-interferon, and downregulating cytokines associated with the Th2 pathway, said cytokines comprising interleukin 4, interleukin 5, interleukin 6 and interleukin 10, said method comprising contacting a cell culture comprising $CD2^+$ cells with an amount of a tryptophan-containing compound effective to increase interferon production, wherein the tryptophan-containing compound is:

(1) L-Xaa-L-Trp

(2) a cyclic form of L-Xaa-L-Trp;

(3) a linear or cyclic polymer of L-Xaa-L-Trp, the polymer having no more than 20 or no more than 10 amino acids; or

(4) a derivative of any of the foregoing compounds which hydrolyses in aqueous solution into any of the foregoing compounds, and

wherein Xaa is Glu, Ile or Leu.

2. The method of claim 1 wherein the CD2+ cells are human cells and the tryptophan-containing compound is L-Xaa-L-Trp or a pharmaceutically acceptable salt thereof.

3. The method of claim 2, wherein the cell culture comprises Th0 cells and whereby the cell culture, after said contacting, comprises a higher percentage of Th1 cells than the culture before said contacting.

4. The method of claim 2, wherein the cell culture comprises Th2 cells and whereby the cell culture, after said contacting, comprises a higher percentage of Th1 cells than the culture before said contacting.

5. The method of claim 2, wherein the cell culture comprises Th1 cells and whereby the cell culture, after said contacting, comprises a higher percentage of over-functionalized Th1 cells than the culture before said contacting.

6. The method of claim 2, wherein the cell culture comprises NK cells and whereby the cell culture, after said contacting, comprises a higher percentage of Th1 cells than the culture before said contacting.

7. The method of claim 2, wherein the cell culture is a culture of bone marrow cells or peripheral blood cells.

8. The method of claim 7, wherein the cell culture is enriched for $CD2^+$ cells.

9. The method of claim 2, wherein the effective amount of the tryptophan-containing compound per $CD2^+$ cell is from about 1×10^6 to about 4×10^6 molecules.

10. The method of claim 2, wherein the tryptophan-containing compound is L-Glu-L-Trp or a pharmaceutically acceptable salt thereof.

11. The method of claim 2, wherein the tryptophan-containing compound is L-Ile-L-Trp or a pharmaceutically acceptable salt thereof.

12. The method of claim 2, wherein the tryptophan-containing compound is L-Leu-L-Trp or a pharmaceutically acceptable salt thereof.

13. A method of producing a composition comprising a cocktail of cytokines associated with the Th1 pathway comprising the steps of:

(a) contacting a cell culture comprising $CD2^+$ cells with an amount of a tryptophan-containing compound effective to increase interferon production, wherein the tryptophan-containing compound is:

(1) L-Xaa-L-Trp

(2) a cyclic form of L-Xaa-L-Trp;

(3) a linear or cyclic polymer of L-Xaa-L-Trp, the polymer having no more than 20 or no more than 10 amino acids; or

(4) a derivative of any of the foregoing compounds which hydrolyses in aqueous solution into any of the foregoing compounds, and

wherein Xaa is Glu, Ile or Leu; and

(b) collecting a supernatant comprising the cocktail of cytokines from the cell culture.

14. The method of claim 13 wherein the CD2+ cells are human cells and the tryptophan-containing compound is L-Xaa-L-Trp or a pharmaceutically acceptable salt thereof.

15. The method of claim 14, wherein said cell culture comprises cells taken from healthy individuals.

16. The method of claim 14, wherein said cell culture comprises Th0 cells and whereby said cell culture, after said contacting, comprises a higher percentage of Th1 cells than said culture before said contacting.

17. The method of claim 14, wherein said cell culture comprises Th2 cells and whereby said cell culture, after said contacting, comprises a higher percentage of Th1 cells than said culture before said contacting.

18. The method of claim 14, wherein said cell culture comprises Th1 cells and whereby said cell culture, after said contacting, comprises a higher percentage of over-functionalized Th1 cells than said culture before said contacting.

19. The method of claim 14, wherein said cell culture comprises NK cells and whereby said cell culture, after said contacting, comprises a higher percentage of Th1 cells than said culture before said contacting.

20. The method of claim 14, wherein said cell culture is a culture of bone marrow cells or peripheral blood cells.

21. The method of claim 20, wherein said cell culture is enriched for $CD2^+$ cells.

22. The method of claim 14, wherein the effective amount of the tryptophan-containing compound per $CD2^+$ cell is from about 1×10^6 to about 4×10^6 molecules.

23. The method of claim 14, wherein the tryptophan-containing compound is L-Glu-L-Trp or a pharmaceutically acceptable salt thereof.

24. The method of claim 14, wherein the tryptophan-containing compound is L-Ile-L-Trp or a pharmaceutically acceptable salt thereof.

25. The method of claim 14, wherein the tryptophan-containing compound is L-Leu-L-Trp or a pharmaceutically acceptable salt thereof.

26. The method of claim 14, further comprising enriching the supernatant for said cocktail of cytokines.

27. A composition comprising a cocktail of cytokines produced by a method comprising the steps of :

(a) contacting a cell culture comprising $CD2^+$ cells with an amount of a tryptophan-containing compound effective to increase production of cytokines associated with the Th1 pathway and decrease production of cytokines associated with the Th2 pathway, wherein the tryptophan-containing compound is:

(1) L-Xaa-L-Trp

(2) a cyclic form of L-Xaa-L-Trp;

(3) a linear or cyclic polymer of L-Xaa-L-Trp, the polymer having no more than 20 or no more than 10 amino acids; or

(4) a derivative of any of the foregoing compounds which hydrolyses in aqueous solution into any of the foregoing compounds, and

wherein Xaa is Glu, Ile or Leu; and

(b) collecting supernatant from said culture, whereby said culture comprises said cocktail of cytokines, and wherein said cocktail is characterized by at least about 25% greater activity in the woodchuck hepatitis model than an equivalent amount of a purified interferon.

28. The method of claim 27 wherein the CD2+ cells are human cells and the tryptophan-containing compound is L-Xaa-L-Trp or a pharmaceutically acceptable salt thereof.

29. The composition of claim 28, wherein said cocktail has an activity of at least about 50% in the woodchuck hepatitis model.

30. The composition of claim 28, further comprising a pharmaceutically acceptable carrier.

31. The composition of claim 28, wherein said cocktail is lyophilized.

32. A composition comprising a cocktail of cytokines which, compared with a cocktail produced by cultured, untreated peripheral white blood cells, comprises a statistically significant increase in the amount of at least one cytokine associated with the Th1 pathway and a statistically significant decrease in the amount of at least one cytokine associated with the Th2 pathway.

33. A method for treating a subject suffering from a pathological condition responsive to interferon treatment selected from the group consisting of viral infection, bacterial infection, fungal infection, benign tumor, malignant tumor, autoimmune disease, degenerative inflammatory condition and asthma, said method comprising the step of administering a pharmacologically effective amount of a composition to the subject; said composition comprising a cocktail of cytokines produced by a method of:

(a) contacting a cell culture comprising $CD2^+$ cells with an amount of a tryptophan-containing compound effective to increase production of cytokines associated with the Th1 pathway and decrease production of cytokines associated with the Th2 pathway, wherein the tryptophan-containing compound:

(1) L-Xaa-L-Trp

(2) a cyclic form of L-Xaa-L-Trp;

(3) a linear or cyclic polymer of L-Xaa-L-Trp, the polymer having no more than 20 or no more than 10 amino acids; or

(4) a derivative of any of the foregoing compounds which hydrolyses in aqueous solution into any of the foregoing compounds, and wherein Xaa is Glu, Ile or Leu; and

(b) collecting supernatant comprising the cocktail of cytokines from the cell culture; wherein the cocktail is characterized by at least about 25% greater activity in the woodchuck hepatitis model than an equivalent amount of a purified interferon.

34. The method of claim 33 wherein the CD2+ cells are human cells and the tryptophan-containing compound is L-Xaa-L-Trp or a pharmaceutically acceptable salt thereof.

35. The method of claim 34, wherein said cell culture comprises cells taken from the subject.

36. The method of claim 34, wherein said subject is a human.

37. The method of claim 34, wherein said cell culture comprises cells taken from a second subject, wherein the second subject does not suffer from the pathological condition.

38. The method of claim 34, wherein said cell culture comprises cells from bone marrow or peripheral blood.

39. The method of claim 38, wherein said cell culture is enriched for $CD2^+$ cells.

40. The method of claim 34, wherein the tryptophan-containing compound is L-Glu-L-Trp.

41. The method of claim 34, wherein the tryptophan-containing compound is L-Ile-L-Trp.

42. The method of claim 34, wherein the tryptophan-containing compound is L-Leu-L-Trp.

43. The method of claim 34, further comprising enriching the supernatant for said cocktail of cytokines.

44. A method for treating a subject suffering from a pathological condition selected from the group consisting of viral infection, bacterial infection, fungal infection, benign tumor, malignant tumor, autoimmune disease, degenerative inflammatory condition and asthma, said method comprising the steps of:

(a) contacting a cell culture comprising $CD2^+$ cells with an amount of a tryptophan-containing compound effective to increase production of

cytokines associated with the Th1 pathway and decrease production of cytokines associated with the Th2 pathway, wherein the tryptophan-containing compound is:

(1) L-Xaa-L-Trp

(2) a cyclic form of L-Xaa-L-Trp;

(3) a linear or cyclic polymer of L-Xaa-L-Trp, the polymer having no more than 20 or no more than 10 amino acids; or

(4) a derivative of any of the foregoing compounds which hydrolyses in aqueous solution into any of the foregoing compounds, and

wherein Xaa is Glu, Ile or Leu; and

(b) administering a pharmacologically effective amount of the cell culture to the subject.

45. The method of claim 44 wherein the CD2+ cells are human cells and the tryptophan-containing compound is L-Xaa-L-Trp or a pharmaceutically acceptable salt thereof.

46. The method of claim 45, wherein said cell culture comprises cells taken from said subject.

47. The method of claim 46, wherein said cell culture comprises cells from bone marrow or peripheral blood.

48. The method of claim 47, wherein said cell culture is enriched for $CD2^+$ cells.

49. The method of claim 46, wherein said subject is immunosuppressed.

50. The method of claim 45, wherein said cell culture comprises cells taken from an individual who does not suffer from the condition.

51. The method of claim 50, wherein said cell culture is encapsulated.

52. The method of claim 45, wherein the tryptophan-containing compound is L-Glu-L-Trp or a pharmaceutically acceptable salt thereof.

53. The method of claim 45, wherein the tryptophan-containing compound is L-Ile-L-Trp or a pharmaceutically acceptable salt thereof.

54. The method of claim 45, wherein the tryptophan-containing compound is L-Leu-L-Trp or a pharmaceutically acceptable salt thereof.

55. The method of claim 45, further comprising enriching the supernatant for said cocktail of cytokines.

56. A method for identifying an optimal therapeutic regimen for a subject with a tumor, said method comprising:

(a) cocultivating a plurality of cultures of tumor cells from said subject and a population of white blood cells comprising $CD2^+$ cells from said subject;

(b) contacting each of the plurality of cultures with a different regimen of a tryptophan-containing compound, wherein the tryptophan-containing compound is:

(1) L-Xaa-L-Trp

(2) a cyclic form of L-Xaa-L-Trp;

(3) a linear or cyclic polymer of L-Xaa-L-Trp, the polymer having no more than 20 or no more than 10 amino acids; or

(4) a derivative of any of the foregoing compounds which hydrolyses in aqueous solution into any of the foregoing compounds, and

wherein Xaa is Glu, Ile or Leu;

(c) measuring the rate of growth of the tumor cells in each the cultures; and

(d) determining the regimen that provides the optimal therapeutic regimen whereby said optimal therapeutic regimen is characterized by inhibition of growth of said tumor cells.

57. The method of claim 56 wherein the CD2+ cells are human cells and the tryptophan-containing compound is L-Xaa-L-Trp or a pharmaceutically acceptable salt thereof.

58. The method of claim 57, wherein the subject is a human.

59. The method of claim 57, wherein the tumor cells are from a solid tumor.

60. The method of claim 57, wherein the tumor cells are hematopoietic in origin.

61. The method of claim 50, wherein the tumor cells and white blood cells are separated by a membrane, wherein the membrane is permeable to compounds with a molecular weight of less than about 100,000 Daltons.

62. The method of claim 50, wherein the $CD2^+$ cells are from bone marrow of the subject.

63. The method of claim 57, wherein at least about 5 differing amounts of the tryptophan-containing compound are added to the plurality of cultures of tumor cells and $CD2^+$ cells.

64. The method of claim 63, wherein the differing amounts of the tryptophan-containing compound are given at least about once per day.

65. The method of claim 57, wherein the tumor cells and $CD2^+$ cells are cocultivated for at least about 5 days.

66. The method of claim 57, wherein the tryptophan-containing compound is L-Glu-L-Trp or a pharmaceutically acceptable salt thereof.

67. The method of claim 57, wherein the tryptophan-containing compound is L-Ile-L-Trp or a pharmaceutically acceptable salt thereof.

68. The method of claim 57, wherein the tryptophan-containing compound is L-Leu-L-Trp or a pharmaceutically acceptable salt thereof.

69. The method of claim 57, wherein the inhibition of tumor cell culture growth is at least about 3-fold.